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Inhibition of genotoxicity during chemoprevention with chlorophyllin and purified chlorophyll from spinach in a mouse model of colon carcinogenesis

Spinach leaves, containing several active components, including flavonoids, exhibit anti-oxidative, anti-proliferative, and anti-inflammatory properties in biological systems. The current study examines the chemopreventive properties of chlorophyllin (CHL) and chlorophyll (Chl) in the colon carcinogenesis model. Male mice were fed purified diets incorporated with either 1000, 2000 p.p.m. CHL, or 1000, 2000 p.p.m. Chl.

Our results of chromosomal aberrations (CA) induced by colon carcinogenic/the colon carcinogen of mice cells showed that the aberrant types were both structural and numerical types. It was clearly indicated that deletion and centromeric attenuation as structural types besides the polyploidy as numeric types were the most frequent types of aberration induced after being treated with cyclophosphamide as a colon carcinogenic agent.

The inhibitory effect of chlorophyllin (CHL) and chlorophyll (Chl) of CA or sister chromatid exchanges (SCE's) was found to be time dependent with the used concentrations and that the percentage of abnormalities reached the control value after 30 days. This is a demonstration that CHL can provide potent chemo protection in a colon carcinogenesis model and support a mechanism involving complex-mediated reduction of carcinogen uptake. In summary, CHL appears to be effective in the reduction of colon cancer risk.

Key Words: Genotoxicity, chlorophyllin, chlorophyll, colon carcinogenesis, chromosomal aberrations, sister chromatid exchange

INTRODUCTION

The study of experimental colon carcinogenesis in rodents has had a remarkably long history, dating back almost 80 years {1}. The earlier study has demonstrated tumorigenesis in the forestomach and intestine of mice following feeding with the polycyclic aromatic hydrocarbon, methylcholanthrene {2}. The feeding radioactive yttrium to rats induced a high proportion of colon tumors has been reported {3}.

Naturally occurring micronutrients in food have been found to have chemopreventive effects, perhaps supporting in part the conclusions from epidemiologic studies that consumption of fresh fruits and vegetables reduces cancer risk {4, 5}.

Spinach leaves containing several active components including flavonoids exhibit antioxidative, antiproliferative, and antiinflammatory properties in biological systems. Spinach extracts have been demonstrated to exert numerous beneficial effects, such as chemo- and central nervous system protection and anticancer and antiaging functions {6}.

The cancer chemopreventive properties of CHL and Chl *in vivo* were first demonstrated in the aflatoxin B1 (AFB1) hepatocellular carcinoma (HCC) in rainbow trout model {7, 8} and later in a rodent model {9}. Physical complications of carcinogen reduce bioavailability to target organs {10, 11}. A dose of 100–300 mg of CHL given with meals for only 3 months was effective at reducing the urinary biomarker of AFB1-dependent DNA adduction by more than half {12}. The cost of CHL a day is low with no significant side effects being reported, making it extremely attractive for intervention due to the high rate of compliance.

It has been difficult to conduct cancer chemoprevention studies *in vivo*, mainly due to prohibitive costs and chemical instability. A counter-current chromatography method was recently reported {13}, enabling the production of 23 g of highly pure Chl a/b from 90 Kg of spinach leaves in a single run, in addition to demonstrating chemoprevention against AFB1-dependent HCC, in both trout and rat {9},

MATERIALS AND METHODS

Animals

We investigated the ability of chlorophyll (1000, 2000 mg/kg bw) and chlorophyllin (1000, 2000 mg/kg BW) pretreatment three times per week for three successive weeks to inhibit mutations induced by intraperitoneal injection of a single dose of 40 mg/kg BW of cyclophosphamide (CP). Forty male albino mice were divided into four groups: control non-treated group, CP-treated group, chlorophyll-CP-treated group, and chlorophyllin-CP-treated group

Chemicals

CHL and CP were purchased from Sigma Chemical Co. (St Louis, MO). The chlorin content of CHL was based on the manufacturer's assay of 4.5% copper and assertion that all copper was present as copper chlorins. The semipurified diets, Chl was prepared as described below.

Preparation of Chl

The Chl used in this study was extracted from baby spinach purchased from local organic growers. A detailed description of the extraction process can be found elsewhere {14}. Briefly, after removal of stems, the leaves were washed with cold water, freeze dried, washed twice with petroleum ether (boiling point 30–60 °C) and solids were extracted twice using methanol : petroleum ether (3:1, vol/vol). Combined extracts were transferred to a separatory funnel and washed with saturated NaCl. A repeat wash of the aqueous layer with petroleum ether was recombined to give the final extract and again washed with saturated NaCl, filtered and evaporated *in vacuo* (30 °C). On average, 30 g of freeze-dried spinach yielded 300 mg of Chl. This Chl extract (90% pure by high-performance liquid chromatography) contained trace amounts of other pigments (carotenoids), as well as some oils, fats and waxes derived from the spinach leaves.

Preparation of solutions

CHL is virtually insoluble in corn oil. Thus, CHL solutions were prepared and diluted to the administered concentration in tricaprylin (TCP) gavage vehicle.

Animals and treatment protocols

Eight-week-old male mice (Animal house of National Research Center) were housed at the Laboratory. Mice were allowed to acclimate for 1 week at 20 ± 1 °C and 50 ± 10% humidity, with a light–dark cycle of 12 h in isolated cages. Mice were fed rat chew *ad libitum*.

Chromosomal Aberration Assay

Five Swiss mice in each group were given 1000 and 2000 mg CHI/kg b.w. or 1000 and 2000 mg chl/kg b.w {14}. 3 h prior to Cyclophosphamide (CP) injection (40 mg/kg, i.p.). Controls received vehicle solutions (10 ml/kg).

Samples were harvested 24 h, 7, 14 and 30 days after CP administration. Mice were injected with colchicines 14 µg/kg b.w.) 2 h prior sacrification. Bone marrow cells were well flushed from both femora and collected with 0.075 M Kcl as a hypotonic solution. Chromosomal preparation was done according to Yosida and Amano {15}; fixation, spreading on clean slides, air drying and staining with 7% Giemsa stain. Scoring was at least 100 metaphases/ mouse.

Sister Chromatid Exchanges

Chromosomes for sister chromatid exchange (SCE's) were prepared according to Allen {16} with some modifications. 22 h post 5-bromodeoxyuoridin (BrdU) tablet implantation, colchicines (14 µg kg^{-1} b.w.) was injected i.p. Two hours later, the mice were sacrificed. Chromosomes were prepared as previously mentioned. Staining was performed as Perry and Wolff {17} using fluorescence plus Giemsa (FPG) technique. About 25 well spread metaphases were scored and analyzed per animal.

Statistics.

The data obtained were evaluated by Student's t-test analysis, in which all the data were compared to the negative control. P<0.5 is considered significant to the negative control and P< 0.01 is considered highly significant according to the –ve control.

RESULTS

The induced chromosomal abnormalities of bone marrow cells of colon carcinogenic mice were recorded 21.43 ± 1.2 as a highly significant effect (Table 1). Administration of colon carcinogenic mice with CHL or chl affected a significant decrease in the chromosomal aberrations, as demonstrated by Table 1. *In vivo* experiments show that treatment of colon carcinogenic mice with CHL (1000, 2000 ppm) or chl (1000, 2000 ppm) induced a reduction of chromosomal aberrations. The significant effect appears after 1, 7 and 14 days as significant values, while after 30 days, the reduction of the chromosomal abnormalities reached the control value post treatment of CHL or chl as illustrated in Table 1. Treatment with CHL (1000, 2000 ppm) to the colon carcinogenic mice has a reducing significant effect. The optimum reduction that occurred after 30 days recorded 4.0 ± 0.76 and 3.71 ± 0.98 % respectively, compared to the control value (3.33 ± 0.12), (Table 1). Similar results were obtained with the treatment of colon carcinogenic mice with chl (1000, 2000 ppm). In relation to time, the chromosomal aberrations were reduced 1, 7, and 14 days post treatment, where the percentage of abnormalities reached the control value after 30 days and recorded 4.0 ± 1.09 and 3.43 ± 0.67 respectively as shown in Table 1. Control values were obtained from 200 cells of 4 untreated (negative control) mice and the mean number of SCE's was recorded as 0.36%. Colon carcinogenic mice recorded a significant increase of SCE's frequency which was 2.16%. Administration of both 2 doses of CHL (1000, 2000 ppm) causes a significant decrease of SCE's which were recorded as 1.16 % and 1.12% respectively after one day and reached the non-significant value after 30 days recording 0.45% and 0.38% respectively (Table 2). Chl administration (with the 2 doses) caused more frequent reduction of SCE's than CHL treatment. The reductions of SCE's recorded were 1.13 and 1.03 % respectively after one day. While after 14 days post treatment of the high dose (2000 ppm), the incidence of SCE's recorded was 0.42% which was nearly as same as the control value (Table 2).

Reduction of SCE's recorded was nearly the control values (0.40% and 0.37%) after one month of treatment of either 1000, 2000 ppm of CHL or chl (Table 2)

DISCUSSION

Exposure to chemicals in the environment, including tobacco smoke, has been associated with the increase in incidences of disease, malformations or behavior in offspring {18}. A number of chemicals have been shown to be colon carcinogens in rodent models {19} and epidemiology studies suggest that this phenomenon occurs in exposed human populations as well {18, 20}.

To initiate the development of cancer, some chemicals (procarcinogens) must first be metabolized to active carcinogens that are capable of damaging DNA or other critical molecules in susceptible tissues. Since enzymes in the cytochrome P450 family are required for the activation of some procarcinogens, inhibition of cytochrome P450 enzymes may decrease the risk of some types of chemically induced cancers {21}. *In vitro* studies indicate that chlorophyllin may decrease the activity of cytochrome P450 enzymes {22, 23}. Phase II biotransformation enzymes promote the elimination of potentially harmful toxins and carcinogens from the body. Limited data from animal studies indicate that chlorophyllin may increase the activity of the phase II enzyme, quinone reductase {24}.

Chromosomal aberrations (CA) contribute to cancer development in humans and experimental animals {25-28}, and elevated lymphocyte CA and SCE frequencies have been shown to be biomarkers of cancer risk within a population of healthy subjects. The use of SCE as a surrogate for CA is supported by a number of validation studies showing a strong correlation between SCE and CA frequencies within the same cell population {29}. In experimental animals, induction of CA or SCE in appropriate target cells following defined exposures is considered a biomarker of genotoxic exposure and predictive of an agent's potential to induce cancer.

Our results of chromosomal aberrations induced by colon carcinogenic/the colon carcinogen of mice cells showed that the aberrant types induced were both structural and numerical types. It was clearly indicated that deletion and centromeric attenuation as structural types besides the polyploidy as numeric types were the most frequent types of aberration induced after being treated with cyclophosphamide as a colon carcinogenic agent.

Breinholt,V. et al, demonstrated the chemopreventive potential of both CHL and Chl in the trout model and in the rat with AFB1 and DBP as the carcinogen {30, 31}. A preliminary clinical intervention trial in China, where dietary AFB1 exposure is high and HCC represents the major cause of cancer mortality, showed significant protective effects of CHL tablets taken orally at mealtime {32}. Results to date point to the importance of simultaneous coadministration of

CHL with the carcinogen support the complications theory for chemoprevention {33, 34}.

Chlorophyll and chlorophyllin are able to form tight molecular complexes with certain chemicals known or suspected to cause cancer, including polycyclic aromatic hydrocarbons found in tobacco smoke {35}, some heterocyclic amines found in cooked meat {36}, and aflatoxin-B_1 {37}. The binding of chlorophyll or chlorophyllin to these potential carcinogens may interfere with gastrointestinal absorption of potential carcinogens, reducing the amount that reaches susceptible tissues{38}.

Chlorophyllin can neutralize several physically relevant oxidants *in vitro* {39,40}, and limited data from animal studies suggest that chlorophyllin supplementation may decrease oxidative damage induced by chemical carcinogens and radiation {41,42}.

A recent study showed that human colon cancer cells undergo cell cycle arrest after treatment with chlorophyllin {43}. The mechanism involved inhibition of ribonucleotide reductase activity. Ribonucleotide reductase plays a pivotal role in DNA synthesis and repair, and is a target of currently used cancer therapeutic agents, such as hydroxyurea {43}. This provides a potential new avenue for chlorophyllin in the clinical setting, sensitizing cancer cells to DNA damaging agents.

In summary, CHL which is inexpensive and appears to lack toxicity in humans was demonstrated to be effective in the reduction of colon cancer risk. This protection was evident even with tumors that appeared well into adult life and is a further example of the 'fetal basis of disease'.

Chemopreventive strategies that begin early in development have the potential to reduce the suffering associated with cancer, and perhaps other chronic diseases.

In any event, chlorophyllin and chlorophyll have proven to be interesting investigational compounds for studying chemoprevention of carcinogenesis. It is hoped that they will ultimately provide some avenues of cancer protection in humans.

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Table 1: Percentage of chromosomal aberrations and types of aberrations induced with CHL and chl on bone marrow cells of colon carcinogenic mice

Treatment	Sample time (days)	Meataphases with: Scored metaphases	Del	Centr.atten.	Chromatid gap	Chrom.break	Polyploid	Total	Mean % S.E.
-ve Control (non-treated)	0	300	3	2	4	1	0	10	3.33 ± 0.12
Infected mice with cancer	0	350	22	17	11	9	16	75	21.43 ± 1.2 **
Treated carcinogenic mice with CHL									
1000 ppm	1	350	20	16	9	5	13	63	18.1 ± 0.92 **
	7	350	18	11	7	4	11	51	14.5 ± 1.1 **
	14	350	12	7	3	3	7	32	9.14 ± 0.96 **
	30	350	4	4	3	2	1	14	4.00 ± 0.76
2000 ppm	1	350	13	10	3	6	8	40	11.43 ± 1.08 **
	7	350	11	13	3	6	5	38	10.86 ± 0.41 **
	14	350	7	5	3	5	4	24	6.86 ± 1.02 *
	30	350	4	3	3	2	1	13	3.71 ± 0.98
Treated carcinogenic mice with Chl									
1000 ppm	1	350	17	14	3	7	12	53	15.14 ± 0.67 **
	7	350	16	12	4	6	7	47	13.43 ± 0.81 **
	14	350	10	8	4	5	4	31	8.86 ± 1.3 **
	30	350	4	3	3	2	2	14	4.00 ± 1.09
2000 ppm	1	350	15	11	7	4	10	47	13.43 ± 0.92 **
	7	350	10	9	3	8	5	35	10.0 ± 1.1 **
	14	350	6	4	2	6	4	22	6.29 ± 0.84 *
	30	350	3	3	3	2	1	12	3.43 ± 0.67

Abbreviations: Del. : Deletion; Centr. Atten. : Centromeric attenuation; CHL : Chlorophyllin; Chl : Chlorophyll
Number of mice – 5 per each treatment.
Significancy comparing with –ve control * Significant at ($p < 0.01$). ** Significant at $p < 0.05$)

Table 2: Frequency of sister chromatid exchanges (SCE's) induced after treatment with CHL and Chl on colon carcinogenic mouse bone marrow cells.

Treatment	Sample time (days)	Scored metaphases	Scored SCE's				SCE's /metaphase Mean %
			S	D	T	More than T	
Negative Control (non-treated)	0	200	59	11	2	0	0.36
Infected mice with cancer	0	125	162	43	38	27	2.16 **
Treated carcinogenic mice with CHL							
1000 ppm	1	125	90	26	13	16	1.16 **
	7	125	86	24	10	12	1.06 **
	14	125	77	22	11	9	0.95 **
	30	125	40	9	4	3	0.45
2000 ppm	1	125	88	26	11	15	1.12 **
	7	125	80	24	9	10	1.00 **
	14	125	62	20	8	9	0.79 **
	30	125	35	8	2	2	0.38
Treated carcinogenic mice with Chl							
1000 ppm	1	125	90	25	12	14	1.13 **
	7	125	84	24	9	12	1.03 **
	14	125	71	20	9	8	0.86 *
	30	125	36	8	4	2	0.40
2000 ppm	1	125	81	25	12	11	1.03 **
	7	125	76	22	9	9	0.93 **
	14	125	36	12	2	3	0.42
	30	125	33	10	2	1	0.37

- Significancy comparing with negative control

** Significant at 0.01 level

* Significant at 0.05 level